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Two Stilbene Synthase Genes from Japanese Red Pine (*Pinus densiflora*)*¹

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Abstract—Stilbene synthase gene (*sts*) was efficiently amplified by PCR from genomic DNA of Japanese red pine (*Pinus densiflora*). We found that the PCR product is a mixture of, at least, two *sts* genes. Both of the *sts* genes are quite similar in their sequences but are different in length of their introns. They carried respective active sites of the enzyme and expressed simultaneously in the roots but not in the hypocotyls. Together with those findings described in the text, we concluded that the two *sts* genes characterized here are isogenes, but not allogene nor pseudogene.

Keywords: stilbene synthase gene, iso-genes, gene expression, pine seedlings, *Pinus densiflora*

Introduction

Stilbene synthase genes (*sts*) have been, so far, cloned from grape¹⁾, peanuts²⁾, scots pine³⁾ and slash pine⁴⁾. Their sequences are quite similar to those of chalcone synthase genes (*chs*). Protein engineering in both of the enzymes, besides the sequence analyses and phylogenic distribution, strongly suggested that the former genes (*sts*) are derived from the latter genes (*chs*)⁵⁾. The gene products have their enzyme activities as a dimer in nature⁶⁾.

Stilbene derivatives, products for stilbene synthase, are usually abundant in heartwood and are mainly distributed in Pinaceae, Betulaceae, Moraceae, Leguminosae, Myrtaceae⁷⁾, which indicates no direct correlation to the phylogenic evolution. They show wide range of physiological activities, e.g., anti-fungal, anti-tumor and allergic activities. In pine trees, nematocidal activity for pinosylvin monomethylether⁸⁾ is notable in the viewpoint of the Japanese pine tree that is heavily suffered from pine wilt diseases⁹⁾. The stilbene synthase may work as a defense factor in pine trees against the diseases when the gene is active. We have already reported that stilbene synthase gene (*sts*) is expressed by the salicylate treatment in the seedlings of *Pinus densiflora*¹⁰⁾, but the gene is not studied in details.

At present paper, we briefly summarize new *sts* genes cloned and characterized from Japanese red pine.

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Materials and Methods

Plant Materials

The seeds of Japanese red pine, *Pinus densiflora*, were purchased from a local market and are stored in a refrigerator until for use. A haploid mega-gametophyte is obtained from a seed coat detached from a corresponding seedling that is grown for 15 days at 28°C under darkness. For RNA extraction, 10 mM of sodium salicylate had sprayed over the seedlings at the 15 days after seeding, the seedlings thus treated were further incubated for 16 hrs.

Extraction of genomic DNA and total RNA

Genomic DNA was directly extracted from the seeds by conventional CTAB method after the nuclei were isolated. The DNA preparation showed only one clear band without smear on an agarose gel and was larger in its size than 23 kbp after electrophoresis. UV spectra of the preparation showed that the ratio of A_{260} to A_{280} is ca. 2.0. Total RNA was extracted and reverse-transcribed by a method described before¹⁰⁾.

Southern hybridization

Genomic DNA (2.5 µg each) was digested with restriction enzymes (*Bam* HI, *Hind* III and *Eco*R I) at 37°C for 5 hrs. No restriction enzyme was also added as a control. Southern analysis was performed in a conventional manner. A positively charged nylon membrane (Boehringer Mannheim Co., Ltd.) was used for the transfer. Prehybridization and hybridization were performed according to the manufacturer's instructions. The membrane was finally washed twice in 0.1x SSC with 0.1% SDS at 68°C for 15 min. DIG Luminescent Detection Kit for nucleic acid (Boehringer mannheim Co., Ltd) was used for detection of the DNA hybridized. The probe is a *sts* fragment (0.9 kbp) labeled by using PCR DIG Labeling Kit (Boehringer Mannheim Co., Ltd.).

T-A Cloning of PCR products

The PCR products were directly cloned into a plasmid pGEM-T Easy Vector (Promega Co., Ltd.). Then, it was mixed with a competent JM109 stain of *Escherichia coli* and electroporated at 2.4 KV, 4 KΩ. The transformed culture was incubated with 1 ml of SOC medium at 37°C for 1 hr. It plated onto LB plate with ampicillin/isopropylthio-β-D-galactoside (IPTG)/5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal). The plates were incubated overnight at 37°C and clones were selected by blue/white screening.

PCR and Determination of base sequences

PCR was performed basically the same procedure described before¹⁰⁾. The primer pairs are 21 mers and the *sts* sequence from *Pinus sylvestris* (Accession No. X60753 in GenBank) was used as a reference. Dye Terminator Cycle Sequencing FS Ready Reaction

Kit (PERKIN ELMER Co., Ltd.) and ABI PRISMTM 377 DNA Sequencer (PERKIN ELMER Co., Ltd.) was mainly used for sequence analyses.

Results and Discussion

Southern analyses indicated that stilbene synthase gene (*sts*) forms a small gene family in *Pinus densiflora*. Besides *sts*, however, chalcone synthase gene (*chs*) is also detectable in the analysis because of their highly homologous sequences. Thus, PCR primers were carefully designed exclusively for amplification of *sts* but not for that of *chs*. One point six kbp of the gene fragment was obtained by PCR, which covers more than 95% of the *sts* coding sequence. It was confirmed as *sts* by the direct sequencing without subcloning.

The sequence was clearly readable in the exon whereas it was wobbled and hardly readable in the intron. The failure is probably ascribed to plural *sts* species in the PCR products. First, we have examined if the plural species might be derived from the sample population used. Although a single diploid seed was used for the PCR as a DNA source, the intron has no readable sequences. It indicates that a single DNA source still carries plural *sts* species.

Second, we thought that the failure might be caused by such multiple forms as an allelic gene. Allelic genes are not co-amplified by PCR when haploid genome is used as a template. We expected it from the PCR product amplified from a single haploid megagametophyte. However, the intron was not readable in the haploid amplified products, which still indicates plural *sts* species in the product. The finding suggests no participation of allelic gene in the amplified products.

Finally, we subcloned the PCR product that was amplified from a single haploid megagametophyte and, then, sequenced 6 subclones obtained. All subclones obtained were successfully sequenced even if the region was an intron. We found that they consist of two groups. Both of the sequences are quite similar each other and carry active sites of the enzyme. Both of them carry same length in their exons, but different one in their introns. Since the subclones are derived from genomic DNA, we could not judge yet if they are not pseudogenes.

Then, we subcloned PCR products derived from cDNAs, that are amplified by RT-PCR from mRNA in the seedlings. The three clones sequenced consisted of the same two types as those from the genomic DNA. We also found that both of the *sts* species are simultaneously expressed in roots but not in hypocotyls of the seedlings.

Together with those findings above mentioned, we concluded that the two *sts* species characterized here are isogenes each other, but not allogene nor pseudogene. On the contrary, three genes involved in lignin biosynthesis showed no isogene in *P. taeda*¹¹⁾. It is also noteworthy that the *sts* gene products usually thought to be a homodimer in nature, but both of the *sts* species might form a hetero-dimer as a functional form in the roots.

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